THE METABOLISM OF $4\text{-}^{14}C\text{-}22,25$-BISDEOXYECDYSONE DURING LARVAL
DEVELOPMENT IN THE TOBACCO HORNWORM, MANDUCA SEXTA (L.)

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ABSTRACT

Hydroxylation and conjugation were the principal routes of
metabolism for $4\text{-}^{14}C\text{-}22,25$-bisdeoxyecdysone when fed to tobacco horn-
worms during larval development. The major metabolite of $4\text{-}^{14}C\text{-}22,25$-
bisdeoxyecdysone isolated from frass and also detected in prepupae was
the tetrahydroxy steroid 22-deoxyecdysone. In addition, three $4\text{-}^{14}C\text{-}$
pentahydroxy analogs -- 22-deoxyinokosterone, 22-deoxy-26-hydroxy-
ecdysone, and 22-deoxy-20-hydroxyecdysone -- were isolated and
identified from frass. A hexahydroxy steroid(s) detected in trace
amounts in prepupae was isolated from frass and tentatively identified
as 20-hydroxyecdysone. Several other unidentified radiolabeled
metabolites were also present in frass and prepupae. The conversion
of $4\text{-}^{14}C\text{-}22,25$-bisdeoxyecdysone to $4\text{-}^{14}C\text{-}\alpha$-ecdysone and $4\text{-}^{14}C\text{-}20$-
hydroxyecdysone was at most a minor pathway since only minute amounts
were found in frass or prepupae. The steroid moieties of the
conjugates, in general, represented qualitatively what was found in
the unconjugated form. The metabolic pathways for the labeled
22,25-bisdeoxyecdysone during larval development and the previously
reported pathways for this ecdysone analog are compared and discussed.

INTRODUCTION

In previous communications, we reported on the metabolism of
$^{3}H\text{-}22,25$-bisdeoxyecdysone (I) in relation to two of its biological

effects in insects: the termination of pupal diapause in the tobacco

hornworm, Manduca sexta (L.) (1, 2, 3, 4) and inhibition of ovarian

development in the adult house fly, Musca domestica L. (2, 3, 4). This

synthetic ecdysone analog has also been shown to inhibit larval growth

and development in a number of insect species including the tobacco

hornworm (5). We now wish to report on the metabolism of the $4\text{-}^{14}\text{C-}$ 22,25-bisdeoxyecdysone during larval development in this insect.

III, R = H
IIIa, R = Ac

I   II

IV , R = H
IVa, R = Ac

V , R = H
Va, R = Ac
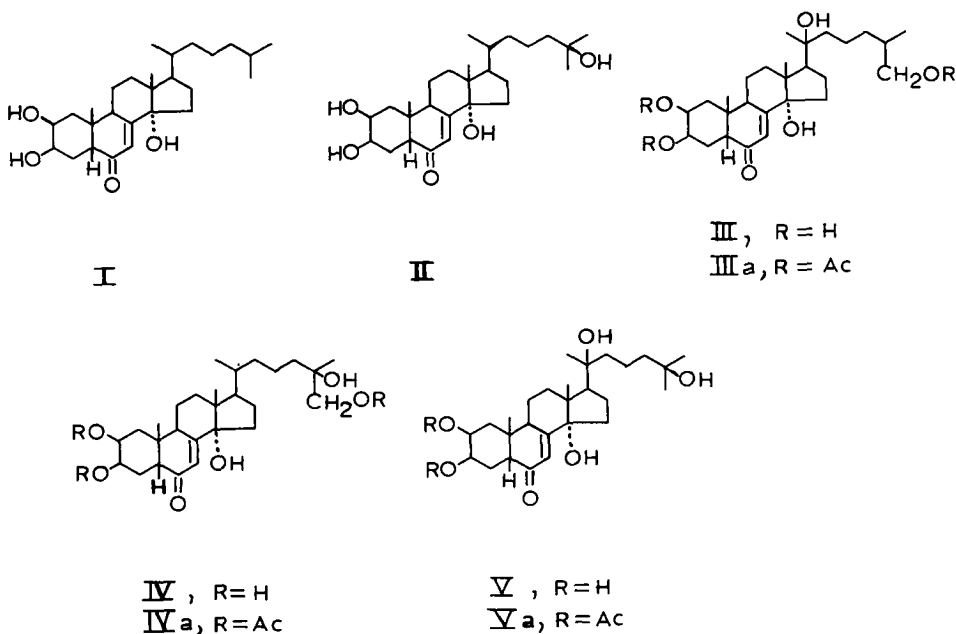
EXPERIMENTAL METHODS AND RESULTS

International Model PR-2 portable refrigerated centrifuge (6) was used to centrifuge extracts. Countercurrent distribution (CCD) was made with a manually operated Craig-Post 60-tube train with 10-ml volumes for the upper and lower phase. Ultraviolet spectra were taken in methanol with a Bausch and Lomb Spectronic 505 spectrophotometer. Developed Silica Gel G thin-layer chromatography (TLC) plates were examined under a Chromato-vue instrument box under short wavelength ultraviolet light for visible zones or spots. Spots were also detected by spraying the plate with 50% sulfuric acid and then heating to 100°. Mass spectra were measured by using a LKB Model 9000 gas chromatography mass spectrometer; the samples were introduced directly into the ionization chamber and the ionization energy was 70 ev. NMR spectra were taken in 50-μl NMR tubes and recorded at 60 Mc with a Varian A-60A NMR Spectrometer equipped with a Varian C-1024 time averaging computer. Deuterated chloroform was used as the solvent for the acetate derivatives, pyridine for the hydroxy steroids, and TMS was the internal NMR standard. All melting points were determined on the Kofler block and are uncorrected.

Labeled Compound. The 4-^{14}C-22,25-bisdeoxyecdysone (I), with a specific activity of 1.4 X 10^6 dpm/mg, was prepared from 4-^{14}C-cholesterol according to procedures used for the syntheses of the unlabeled compound (7). Its radiochemical purity was greater than 98% as determined by column and thin-layer chromatography. For these studies, 18.75 mg of the 4-^{14}C-22,25-bisdeoxyecdysone was diluted with 363.75 mg of the unlabeled compound to give a final observable specific activity of 7.0 X 10^4 dpm/mg. The purity of the unlabeled compound used for dilution was equivalent to that of the labeled ecdysone as determined by column and thin-layer chromatography and spectral analyses (UV, IR, NMR, Mass spectrometry).

Biological Material. Fifty-five newly hatched tobacco hornworm larvae were reared to the early prepupal stage on an artificial diet (8) containing the radiolabeled compound which was incorporated into the diet by coating the compound on the dry dietary components with methanol and allowing the solvent to evaporate under the hood. The concentration of the 4-^{14}C-22,25-bisdeoxyecdysone in the diet was 150 ppm (wet weight), well below the concentration required to severely inhibit development and yet in sufficient quantity to assure the availability of radiolabeled metabolites for study.

The frass, following removal of diet particles, was collected twice daily, pooled in a common container, and kept frozen until extracted. The prepupae were collected and held individually for 1 day in half-pint containers to permit them to evacuate their digestive tracts. At the end of the one-day period the organisms were cleansed with moistened tissue paper, blotted dry, weighed, and held frozen until extracted.

Bioassay. The house fly assay (9) was used to detect molting hormone (MH) activity at certain steps during the fractionation and to assess the activity of purified metabolites after isolation. The compounds were injected into the test insects either as aqueous or as 30% methanolic solutions or if insoluble in these, as 5% hydroxy lecithin emulsions (10).

Extraction, Isolation, and Identification of Labeled Compounds from Frass. The frass (361 g) was homogenized for 5 minutes with 3 ml of methanol/g (wet weight). The homogenate was transferred to 250-ml glass cups and centrifuged at 2500 rpm for 10 minutes. The supernatants were decanted off, and the residues in the centrifuge tubes were pooled and rehomogenized twice with 75% methanol (1.5 ml/g). The extracted residues in the tubes were pooled and radioassayed (50 g, 1.5 X 10^5 dpm total) and then discarded. The supernatants were combined and taken to dryness in vacuum (33g), and the residue was partitioned between 100 ml of water and 50 ml of butanol. After centrifugation, the butanol phase was removed, and the aqueous phase was extracted twice with 25 ml of butanol. The aqueous phase (27 g, 3.3 X 10^5 dpm) was discarded. The butanol extracts were combined,

washed once with 2% sodium carbonate solution, and then rinsed with
water until neutral. The combined butanol extracts were taken to
dryness (1.7 g, 6.0 X 10^6 dpm total). The residue was partitioned
between 100 ml each of 70% methanol and hexane, the phases were
separated, and each phase was re-extracted with 100 ml of the appro-
priate upper or lower phase. The hexane fractions (0.6 g, 8.8 X 10^4
dpm total) were discarded. The 70% methanolic fractions were combined
and taken to dryness (1.1 g, 6.4 X 10^6 dpm total), and the residue was
dissolved in 2.5 ml of methanol and adjusted with benzene to give a
final concentration of benzene-methanol (95:5). The supernatant was
chromatographed on a 20-g column (3.3 X 4.7 cm) of benzene-washed
silicic acid (11) and eluted with increasing concentrations of methanol
in benzene and finally with methanol. The elution pattern and the
distribution of mass and radioactivity are shown in Table 1.

Table 1
Column chromatography a/ of total 4-^{14}C-compounds isolated from frass
of tobacco hornworms reared on a diet containing 4-^{14}C-22,25-bisdeoxy-
ecdysone.

Fraction	Volume (ml)	Mass (mg)	Total dpm (X 10^5)	Percent of total
Ø-MeOH (95:5)	1800	578	36.0	64.1
Ø-MeOH (90:10)	1000	67	4.4	7.8
Ø-MeOH (75:25)	225	41	1.8	3.2
MeOH	225	149	14.0	24.9

a/   Column 3.3 cm (I.D.) X 4.7 cm, 20 g of silicic acid.

The benzene-methanol (95:5) fraction (578 mg, 3.6 X 10^6 dpm, Table
1) which elutes the 22,25-bisdeoxyecdysone (I) was further refrac-
tionated on a column of 14.6 g (2.8 X 4.7 cm) of benzene-washed silicic
acid and eluted with smaller volumes of benzene-methanol (95:5) as
shown in Table 2. When authentic 4-^{14}C-22,25-bisdeoxyecdysone was
chromatographed in this manner, over 93% of the radioactivity was
eluted in fraction 2. Fraction 2 (207 mg, 2.0 X 10^6 dpm, Table 2) was
dissolved in chloroform-methanol (1:1), applied to several TLC plates,
and developed in a solvent system of chloroform-ethanol (8:2) (12).
Extraction of the 22,25-bisdeoxyecdysone zones with methanol yielded
41.5 mg, 1.6 X 10^6 dpm total. The compounds were chromatographed on a
microcolumn of 2.25 g (1.1 X 4.7 cm) of benzene-washed silicic acid and
eluted with benzene-methanol (95:5) and the following fractions were
collected: fraction 1, 10 ml; fraction 2, 15 ml; and fraction 3
through 5, 25 ml. Methanol (25 ml) was used to strip the column.
Fraction 2 from this column contained 32.4 mg, 1.5 X 10^6 dpm. Crystal-

lization of this material to constant physical properties and a
constant specific activity from benzene-methanol and ethyl acetate-
methanol gave 13 mg of crystals as rosettes, m.p. 206 - 208° with dec.,
λ max. 245 nm, ϵ 12,108, specific activity 6.6 X 10^4 dpm/mg, NMR δ 0.75
(18-H), 1.08 (19-H), 1.06, 0.975 (21-H), 0.925, 0.83 (26- and 27-H).
Comparative TLC, NMR and mass spectral analyses confirmed that the
steroid is 22,25-bisdeoxyecdysone (I). The compound was as active as
authentic I (13) in the house fly molting hormone assay.

Table 2
Rechromatography $\underline{a}/$ of the $\emptyset$-MeOH (95:5) fraction from Table 1.

Fraction	Volume (ml)	Mass (mg)	Total dpm (X 10^4)	Percent of total	Compounds Eluted
$\emptyset$-MeOH (95:5)					
1	75	268	0	0	
2	100	207	200.0	56.7	22,25-bisdeoxyecdysone
3	175	33	130.0	36.9	22-deoxyecdysone
4	175	16	4.1	1.2	
5	175	6	9.7	2.7	
MeOH	175	7	8.8	2.5	

$\underline{a}/$ Column 2.8 cm (I.D.) X 4.7 cm, 14.6 g of silicic acid.

Fraction 3 (33 mg, 1.3 X 10^6 dpm, Table 2) from the column was
chromatographed by TLC and developed in the same system as described
for fraction 2. Extraction and radioanalysis of the major zone yielded
21.5 mg, 9.5 X 10^5 dpm total, 82% of the total radioactivity recovered
from the plate. Three crystallizations from benzene-methanol, dilute
methanol, and finally benzene-methanol gave 10.4 mg of rods with
constant physical properties, m.p. 216 - 217° with dec., λ max. 245 nm,
ϵ 11,746, and a constant specific activity of 6.5 X 10^4 dpm/mg (corr.
for a tetrahydroxy steroid). NMR, δ 0.73 (18-H), 1.08, 0.99 (21-H),
1.40 (26- and 27-H) mass spectrum (m/e, rel. intensity) M^+ 448(3),
430(41), 420(16), 412(79), 402(98), 397(57), 384(26), 379(24), 369(20),
357(13), 341(35), 331(28), 327(45), 301(68), 300(45), 283(33), 277(31),
276(29), 249(100), 231(52), 223(37), 213(26), 201(27), 199(32),
185(32), 173(52), 161(45), 147(63), 145(60), 131(45), 121(56), 109(62),
91(61), 81(77), 69(94), 55(98) with metastables at m/e 385, 367, 362,
266, and 214.5.

The M^+ of 448 indicated a tetrahydroxy compound. The appearance
of the C-26 and 27-methyl resonances as a singlet at δ 1.40 placed the
additional hydroxyl group at C-25; thus, this compound is 22-deoxy-

ecdysone (II). Its R_f was similar to that of synthetic 22-deoxy-ecdysone. The compound was about one-fourth as active as α-ecdysone (13) in the house fly molting hormone assay.

Seventy-five percent of the benzene-methanol (90:10) fraction (Table 1) was subjected to CCD in the solvent system of cyclohexane-butanol-water, 5:5:10. After 50 transfers, tubes 10 - 21 which would contain 20-hydroxyecdysone were pooled (1.1 mg, 7040 dpm total). Likewise, tubes 28 - 43 which would contain α-ecdysone were combined (14.5 mg, 2.9 X 10⁵ dpm total). When the material from CCD tubes 10 - 21 was chromatographed on a preparative TLC plate in the solvent system of chloroform-ethanol (8:2), about 60% of the radioactivity was found to be associated with the 20-hydroxyecdysone, and its biological activity in the house fly assay was equivalent to that for 20-hydroxyecdysone. Also, the mass spectrum gave fragmentation patterns similar to those of 20-hydroxyecdysone. However, because of the lack of sufficient material for further analyses, the compound was only tentatively identified as 20-hydroxyecdysone.

When the material from CCD tubes 28 - 43 was purified by preparative TLC, about 88% (7.0 mg, 2.4 X 10⁵ dpm total) of the total radioactivity recovered from the plate was associated with a material that had an R_f slightly below that of α-ecdysone. The compound(s) failed to crystallize from ethyl acetate-methanol and, while the mass spectrum of this material indicated a pentahydroxy steroid(s), its complex NMR spectrum indicated a mixture.

When the mixture (3.3 mg, 1.7 X 10⁵ dpm total) was subjected to TLC and developed several times in chloroform-ethanol (9:1), a broad zone (4.7 cm) was visualized under uv light. The zone was arbitrarily divided into three sections -- an upper, middle, and lower section, and each was extracted, radioassayed, and analyzed further by NMR and mass spectroscopy.

The NMR spectrum of the material from the upper section (0.41 mg, 2.0 X 10⁴ dpm) now exhibited a simple NMR spectrum with the methyl resonances at δ 1.12 (18-H), 1.09 (19-H), 1.53 (21-H), and 1.09, 1.02 (27-H). This pentahydroxy steroid is 22-deoxyinokosterone (III), mass spectrum (m/e, rel. intensity), (M⁺ 464) (1), 446(7), 428(4), 413(9), 395(7), 345(12), 327(32), 309(9), 300(23), 287(9), 285(12), 269(11), 267(15), 249(13), 239(11), 231(10), 227(11), 225(12), 211(15), 199(13), 197(11), 185(13), 173(17), 171(15), 145(39), 127(23), 121(20), 109(55), 93(24), 91(25), 81(30), 69(53), 55(47), 43(100).

Its methyl resonances differed from those of inokosterone only in having the C-18 methyl appear upfield at δ 1.12 while that of inokosterone appears downfield at δ 1.22 due to the hydroxyl group at C-22 in inokosterone. The lack of methyl resonance in the region of δ 0.75 for a C-18 methyl group as in the spectrum of compounds I and II also places a hydroxy group at C-20.

The NMR spectra of the material from the middle and lower sections
of the band still indicated a mixture of compounds. The acetates
/pyridine-acetic anhydride (3:1) 43 hr at room temperature/ were pre-
pared of the material from the upper, middle, and lower part of the
zone as well as the acetate derivatives of α-ecdysone, 20-hydroxy-
ecdysone, and inokosterone. Analyses by TLC of the acetate derivative
from the upper part of the zone gave only one component, 22-deoxyinoko-
sterone 2,3,26-triacetate, when developed in the solvent system of
ethyl acetate-benzene (3:1), and its R_f was similar to that of inoko-
sterone 2,3,22,26-tetraacetate. The acetate derivative from the middle
section of the zone showed approximately equal quantities of 22-deoxy-
inokosterone 2,3,26-triacetate, and an acetate derivative that had an
R_f slightly less than that of 20-hydroxyecdysone 2,3,22-triacetate.
The TLC of the acetate derivative from the lower section of the zone
showed about 20% of 22-deoxyinokosterone 2,3,26-triacetate, 45% of
slightly more polar material and about 35% of a component that had an
R_f slightly less than that of 20-hydroxyecdysone 2,3,22-triacetate.
The acetate derivatives from the middle and lower section of the zone
were combined and spotted on a 5 X 20-cm analytical Silica Gel G plate
and developed twice in the solvent system of ethyl acetate-benzene
(3:1). The three zones were separated from the plate, and each was
extracted with acetone. In order of increasing polarity, Zone 3
yielded 257 µg of 4-^{14}C-22-deoxyinokosterone 2,3,26-triacetate (IIIa),
$C_{33}H_{50}O_9$ (M^+ 590) NMR, δ 0.87 (18-H), 1.03 (19-H), 1.25 (21-H), 0.87,
1.0 (27-H), 2.12, 2.06, 2.00 (2-, 26- and 3-OCOCH$_3$, respectively).

Zone 2 yielded 191 µg of a compound that had an R_f slightly less
than that of 22-deoxyinokosterone 2,3,26-triacetate (IIIa) on a Silica
Gel G plate. From the interpretation of its NMR spectrum (δ) 0.68
(18-H), 1.03 (19-H), 0.98, 0.87 (21-H), 1.23 (27-H), 2.11 (2- and 26-
OCOCH$_3$), 2.00 (3-OCOCH$_3$) and mass spectral analyses, this compound is
4-^{14}C-22-deoxy-26-hydroxyecdysone 2,3,26-triacetate (IVa), $C_{33}H_{50}O_9$
(M^+ 590).

Zone 1 yielded 158 µg of a compound that exhibited an R_f slightly
less than that of 20-hydroxyecdysone 2,3,22-triacetate. This compound
is 4-^{14}C-deoxy-20-hydroxyecdysone 2,3-diacetate (Va) $C_{31}H_{48}O_8$ (M^+ -H$_2$O
at m/e 530), NMR, δ 0.85 (18-H), 1.03 (19-H), 1.26 (21-H), 1.26 (26- and
27-H), 2.11, 2.00 (2- and 3- OCOCH$_3$, respectively).

Isolation and Identification of 4-^{14}C-Steroidal Moieties of
Conjugates from Frass. The benzene-methanol (75:25) fraction (41 mg,
1.8 X 10^5 dpm and the methanol fraction (149 mg, 1.4 X 10^6 dpm) of
Table 1 were combined, and an aliquot was rechromatographed on a micro-
column of silicic acid (2.25 g). About 95% of the radioactive
material was eluted in the benzene-methanol (75:25) and methanol
fractions, thus confirming the polarity of these 4-^{14}C-compounds.
Since previous studies had shown that these fractions contained
conjugates principally of sulfates and some glucosides, the remaining
material was incubated with a mixture of sulfatase and β-glucosidase in

30 ml of a buffer solution of 0.2 M sodium acetate-acetic acid for 24 hours at 37° (14). The buffer solution was extracted three times with 1/2 volumes of butanol, and the residue from the butanol extracts (159 mg, 1.5 X 10^6 dpm total) was chromatographed on 14.6 g (2.8 X 4.7 cm) of benzene-washed silicic acid (Table 3). Fraction 2 (57.0 mg, 1.1 X 10^6 dpm, Table 3), which elutes the 22,25-bisdeoxyecdysone, was distributed between two preparative TLC plates and developed with chloroform-ethanol (8:2). Ultraviolet light revealed a visible zone with an R_f similar to that for 22,25-bisdeoxyecdysone (I) and this zone (18.2 mg, 9.0 X 10^5 dpm) possessed 92% of the total radioactivity recovered. Three recrystallizations to constant physical properties and specific activity from dilute methanol, benzene-methanol, and ethyl acetate-methanol gave 10.0 mg of cubelike crystals m.p. 201.5 - 203°, with dec. λ max. 245 nm €11,900, specific activity 6.4 X 10^4 dpm/mg. Comparative TLC, NMR, and mass spectral analyses confirmed this compound to be I.

Table 3

Column chromatographic analyses a/ of Ø-MeOH (75:25) and MeOH fractions (Table 1) from frass following hydrolyses with a sulfatase-β-glucosidase preparation.

Fraction	Volume (ml)	Mass (mg)	Total dpm (X 10^4)	Percent of total	Compounds Eluted
Ø-MeOH (95:5)					
1	75	2.8	0.5	0.3	
2	100	57.0	110.0	75.9	22,25-bisdeoxyecdysone
3	175	7.8	18.0	12.4	22-deoxyecdysone
4	175	1.8	1.1	0.7	
5	175	1.2	1.4	1.0	
6	350	2.0	2.1	1.5	
7	350	1.5	0.8	0.6	
Ø-MeOH (90:10)					
8	175	3.7	1.5	1.0	α-ecdysone,
9	175	3.2	1.3	0.9	other pentahydroxy
10	175	2.9	0.8	0.6	steroids and
11	175	2.4	0.8	0.6	20-hydroxyecdysone
Ø-MeOH (75:25)					
12	175	-	2.7	1.9	Polars
MeOH					
13	175	-	3.8	2.6	Polars

a/   Column 2.8 cm (I.D.) X 4.7 cm, 14.6 g of silicic acid.

Fraction 3 (7.8 mg, 1.8 X 10^5 dpm, Table 3) was purified by preparative TLC, and a zone with an R_f similar to that of 22-deoxy-ecdysone was detected. Extraction of material from this zone (3.5 mg, 1.4 X 10^5 dpm) and crystallization from dilute methanol yielded 1.8 mg of crystals as plates, m.p. 217-219 (with dec.) λ max. 245 nm (€9,500), specific activity 5.1 X 10^4 dpm/mg (corr.).

Its NMR spectrum, with the methyl resonances at δ 0.73 (18-H), 1.08 (19-H), 1.08, 0.99 (21-H), 1.40 (26- and 27-H), and the mass spectrum fragmentation pattern were identical to that of 22-deoxy-ecdysone isolated from frass.

Fractions 8 through 11 (12.2 mg, 4.4 X 10^4 dpm, Table 3) were pooled and fractionated by CCD through 50 transfers. When the compounds from CCD tubes 10 - 20, which contained about 10% of the radioactivity (1.1 mg, 3100 dpm), were analyzed by TLC in the solvent system of chloroform-ethanol (8:2), about 1/10 of the radioactivity was recovered in the 20-hydroxyecdysone area. The major portion of the radioactivity represented more polar metabolites. No further analyses were made with either of these fractions.

When the material from CCD tubes 28 - 45 (8.51 mg, 3.1 X 10^4 dpm) was analyzed by TLC, no definite radioactivity could be accounted for α-ecdysone. However, several zones more polar than α-ecdysone were detected, including a zone for the pentahydroxy steroids. Further analyses by TLC in the solvent system of chloroform-ethanol (9:1) (developed several times) confirmed this zone to be similar to that of the pentahydroxy steroids present in the unconjugated form from frass.

Steroids and Steroid Moieties of Conjugates from Prepupae. The frozen prepupae (353 g) were transferred to a large coarse glass-fritted funnel and rinsed quickly three times with 200 ml each of methanol to remove any external 4-^{14}C-contaminants (1.2 X 10^4 dpm). The prepupae were then homogenized and extracted in the same manner as described for the frass.

When the 70% methanol extractive (628 mg, 4.8 X 10^4 dpm) was chromatographed on silicic acid and eluted in the same manner as shown for frass in Table 1, the benzene-methanol fraction (95:5) eluted 65% of the mass (253 mg), and about 26% of the radioactivity (1.0 X 10^4 dpm) recovered from the column. When this fraction was rechromato-graphed on silicic acid with smaller volumes of benzene-methanol (95:5) as shown for frass in Table 2, about 35% of the mass and 89% of the radioactivity recovered from the column was eluted in fraction 2 (80.6 mg, 5000 dpm) and fraction 3 (2.0 mg, 3000 dpm). TLC analyses of fractions 2 and 3 yielded zones for 22,25-bisdeoxyecdysone and 22-deoxyecdysone, respectively. Mass spectra confirmed the material in fraction 2 as 22,25-bisdeoxyecdysone (I). Although mass spectra showed the compound in fraction 3 to be a tetrahydroxy steroid, it could not be established that the material was 22-deoxyecdysone (II) because of

interfering substances. When the benzene-methanol (90:10) fraction (15.0 mg, 3000 dpm) of the extractives from prepupae were analyzed by TLC in the solvent system of chloroform-ethanol (9:1) and developed several times, the majority of the 4-^{14}C-compound(s) were more polar than that of α-ecdysone and behaved similarly to the pentahydroxy steroids found in frass. Only trace amounts of radioactivity were associated with α-ecdysone and 20-hydroxyecdysone.

The more polar column fractions (121 mg, 2.6×10^4 dpm) contained nearly 67% of the total radioactivity. When this material was hydrolyzed with a sulfatase-β-glucosidase enzyme mixture and the extracted residue fractionated by column as for frass in Table 3, about 50 and 10% of the total radioactivity recovered from the column were eluted in fractions 2 and 3, respectively. TLC and mass spectral analyses of these fractions confirmed the compound in fraction 2 as I and that in fraction 3 as II.

Fractions 8 to 11 eluted 15% of the radioactivity and analyses by TLC did not indicate the presence of either α-ecdysone or 20-hydroxyecdysone, but had an R_f similar to that of the pentahydroxy steroids isolated previously.

DISCUSSION

In our previous studies of the metabolism of radiolabeled 22,25-bisdeoxyecdysone in relation to two of its biological activities-- termination of pupal diapause in the tobacco hornworm and inhibition of ovarian development in the adult house fly--the major metabolic modifications of this ecdysone analog were hydroxylation and conjugation (2, 3, 4). Similar pathways were found in the tobacco hornworm during larval development when 4-^{14}C-22,25-bisdeoxyecdysone was fed in the diet at a level below an inhibitory concentration. Nearly 70% of the total radioactive compounds isolated from frass and about 90% from the early prepupae represented metabolites of 22,25-bisdeoxy- ecdysone, indicating that under the experimental conditions employed, the analog was extensively metabolized by the tobacco hornworm during the period of larval to prepupal development. The major metabolite of 22,25-bisdeoxyecdysone identified from frass, which was also

detected in early prepupae, was the tetrahydroxy steroid 22-deoxy-

ecdysone (II), indicating that hydroxylation at C-25 precedes that at

C-22. The 22-deoxyecdysone has also recently been reported to be a

metabolite of 22,25-bisdeoxyecdysone following injection of this

steroid into the prepupae of both the tobacco hornworm (15) and

Calliphora stygia (16). However, unlike the hornworm larvae, the pre-

pupae of the hornworm and Calliphora efficiently converted the 22-

deoxyecdysone to α-ecdysone, 20-hydroxyecdysone and/or 20,26-dihydroxy-

ecdysone (15, 16). A re-examination of the extracts from our previous

metabolic study with 22,25-bisdeoxyecdysone during termination of

pupal diapause in the hornworm showed that the 22-deoxyecdysone

accounted for less than 1% of the total radioactive compounds. Thus,

at this stage of development, the 22-deoxyecdysone does not accumulate

but is also further metabolized to α-ecdysone, 20-hydroxyecdysone, and

20,26-dihydroxyecdysone.

Clearly, under the experimental conditions described herein, the

conversion of 22,25-bisdeoxyecdysone to α-ecdysone and 20-hydroxy-

ecdysone is, at most, a minor metabolic pathway. Although α-ecdysone

was not detected in frass, three other pentahydroxy steroids -- 22-

deoxyinokosterone (III), 22-deoxy-26-hydroxyecdysone (IV), and 22-

deoxy-20-hydroxyecdysone (V) -- were isolated and identified. In

addition to the pentahydroxy analogs, a hexahydroxy steroid(s) was

also found in small amounts in frass and tentatively identified as

20-hydroxyecdysone. Since all of the major metabolites identified

from frass lack a 22-hydroxyl group, this $4-^{14}$C-hexahydroxy steroid(s)

may be a mixture of a $4-^{14}$C-hexahydroxy steroid which lacks a C-22

hydroxyl group and nonlabeled 20-hydroxyecdysone produced from endoge-

nous precursors. Unfortunately, the minute amount of the material did

not permit further analyses but in the house fly assay, the compound(s)

was as active as authentic 20-hydroxyecdysone.

The early prepupae differed principally from frass in that they

contained what appeared to be trace amounts of α-ecdysone in addition

to the trace amounts of 20-hydroxyecdysone in the non-unconjugated

form. Like frass, the early prepupae also contained a pentahydroxy

steroid fraction but the small quantities of material prevented the

conclusive identification of these metabolites.

Interestingly, the metabolism of 22,25-bisdeoxyecdysone in this

study more closely resembles in complexity the metabolism of 22,25-

bisdeoxyecdysone following the feeding of the compound to the adult

house fly (2, 3, 4) and of 22-deoxyecdysone following its injection

into grasshopper nymphs, Gastrimargus africanus, and larvae of the

blowfly, Sarcophaga bullata (15). In all three, the metabolites were

found to be a complex mixture of steroids other than α-ecdysone, 20-

hydroxyecdysone and/or 20,26-dihydroxyecdysone with the exception of

the blowfly where a small quantity of what appeared to be 20-hydroxy-

ecdysone was detected (15). Although little, if any, 22-deoxyecdysone

was converted to 22-deoxy-20-hydroxyecdysone in either Gastrimargus

nymphs or Sarcophaga larvae, the latter pentahydroxy steroid is a

metabolite of 22,25-bisdeoxyecdysone present in frass of tobacco horn-

worm larvae.

The presence of conjugated metabolites, which accumulated in both

frass and prepupae of the hornworm, was particularly striking in the

early prepupa where over 60% of the total radioactive compounds were
in the form of more polar substances. Previously, we showed sulfates
to be the predominant and glucosides to be the minor conjugate of the
[3]H-molting hormones from tobacco hornworms during pupal-adult develop-
ment (3, 4). In this study, no attempt was made to determine the
relative contribution by the two conjugates. Since our current inter-
est is primarily in the steroidal moieties, the polar fractions from
the frass and prepupae were hydrolyzed with a sulfatase-β-glucosidase
preparation to assure the availability of the steroidal components for
identification. The composition of the steroid moieties of the conju-
gates from frass and early prepupae generally were qualitatively
similar to those in the unconjugated form. In both, unmetabolized 22,
25-bisdeoxyecdysone was the major steroid component and 22-deoxy-
ecdysone was the major ^{14}C-metabolite. Although 4-^{14}C-pentahydroxy
compounds were present, α-ecdysone was not detected in the steroid
conjugate fraction from either frass or prepupa. The apparent absence
of α-ecdysone as a conjugate in frass and prepupae represents another
difference in metabolism from that found during pupal-adult develop-
ment where α-ecdysone was the major steroid conjugate, far exceeding
20-hydroxyecdysone (3, 4) and pointing again to different metabolic
pathways for the ecdysone analog at different times or in the different
stages of development.

How can we reconcile the differences in the metabolism of 22,25-
bisdeoxyecdysone in the different developmental stages of the tobacco
hornworm? The metabolism of the 22,25-bisdeoxyecdysone during pupal-
adult development following termination of pupal diapause (1, 2, 3, 4)

and during prepupal-pupal development (15) was examined at a time when

the biochemical mechanisms necessary for the sequential hydroxylation

of the analog to the ecdysones would be expected to be active in the

tobacco hornworm. Peak titers of molting hormone activity have been

observed in both of these developmental stages of this insect (17).

The relatively low level of conversion of 22,25-bisdeoxyecdysone to

α-ecdysone and 20-hydroxyecdysone in the early prepupa as opposed to

its efficient conversion following injection during the period of

increase in titer of molting hormone activity in the tobacco hornworm

prepupae (15) re-emphasizes the importance of the developmental period

in relation to ecdysone biosyntheses and metabolism. One explanation

for the observed difference in metabolism may be that when fed during

larval development the 22,25-bisdeoxyecdysone is mainly available and

metabolized between molts. At such time, the enzymes necessary for

its sequential hydroxylation may not be present thus resulting in a

complex mixture of hydroxylated metabolites. Another possibility is

that in the early immature stages of development the biosynthetic

pathways to the ecdysones or perhaps even the ecdysones per se may

differ from those currently known. Studies are currently underway to

determine whether one or more of the above mechanisms are responsible

for the observed differences in the metabolism of 22,25-bisdeoxy-

ecdysone in different developmental stages of the tobacco hornworm.

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TRIVIAL AND IUPAC EQUIVALENT NAMES

α-Ecdysone = 2β,3β,14α,22R,25-Pentahydroxy-5β-cholest-7-en-6-one

20-Hydroxyecdysone = 2β,3β,14α,20R,22R,25-Hexahydroxy-5β-cholest-7-en-6-one

22,25-Bisdeoxyecdysone = 2β,3β,14α,-Trihydroxy-5β-cholest-7-en-6-one

22-Deoxyecdysone = 2β,3β,14α,25-Tetrahydroxy-5β-cholest-7-en-6-one

22-Deoxyinokosterone = 2β,3β,14α,20R,26-Pentahydroxy-5β-cholest-7-en-6-one

22-Deoxy-26-hydroxyecdysone = 2β,3β,14α,25,26-Pentahydroxy-5β-cholest-7-en-6-one

22-Deoxy-20-hydroxyecdysone = 2β,3β,14α,20R,25-Pentahydroxy-5β-cholest-7-en-6-one